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A Gene Trap Strategy for Identifying the Gene Expressed in the Embryonic Nervous System

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ABSTRACT—An efficient gene trap strategy was devised for identifying the genes that are expressed in the mouse developing nervous system. Mouse embryonic stem (ES) cell lines that carried independent integrations of a gene trap vector, pS*neo*N*lacZ*A, were allowed to differentiate in a suspension culture system. To select cells containing neurons, astrocytes or neuron-glia precursors, cell lines were immunohistochemically examined with antibodies against neuron-specific proteins (neurofilament protein 150 kD and microtubule associated protein 2), glial fibrillary acidic protein or nestin. Three cell clones (GT3-8, 11 and 12) were immunoreactive to either of the antibodies employed and at the same time positive for β-galactosidase activity. When chimeric embryos were generated by the use of the above 3 cell lines, some cells in their nervous system showed X-gal staining. Thus the major advantage of the present gene trap method lies in its prescreening step of manipulated ES cells prior to generation of chimeric animals. This method holds promise as a useful tool for investigating the genes involved in the development of the nervous system.

INTRODUCTION

During the past decade, there have been great advances in the method to analyze as to how genes affect the developmental processes in various animals. The most efficient and widely used technique is gene targeting where replacement of homologous genes results in morphological, biochemical or physiological defects (Capecchi, 1989; Mansour *et al.*, 1988; Satokata and Maas, 1994; Schneider *et al.*, 1993). The ablation of a given gene is known to cause serious malformations which lead to determine the direct or indirect role of the gene in question. There are, however, only a limited number of genes whose structure is already established. The preparation of altered genes for this method requires the precise knowledge of the gene to be handled.

In an attempt to search for still unidentified genes, the gene trapping method has been developed (Friedrich and Soriano, 1991; Gossler *et al.*, 1989; Joyner *et al.*, 1992; Niwa *et al.*, 1993; Skarnes *et al.*, 1992; Takeuchi *et al.*, 1995). Although this is a prominent method for the identification of various genes, it is laborious to inject all ES cell lines into blastocysts when a number of genes were trapped. So we have attempted to develop a more efficient way to obtain ES cells whose handled genes are expressed in a restricted tissue. In this study we screened gene trapped ES cells that had differentiated *in vitro* before generation of chimeric mice.

Our gene trap vector construct was designed as a dicistronic vector in order to select the ES cell clones containing the trapped gene more easily. The internal ribosomal entry site (IRES) of encephalomyocarditis virus (Jang and Wimmer, 1990) was inserted between the 5' end of the promoterless neomycin phosphotransferase gene (*neo*) and β-galactosidase gene (*lacZ*) (Kim *et al.*, 1992; Takeuchi *et al.*, 1995). When this dicistronic gene was integrated into an active transcriptional unit, activation of both *neo* and *lacZ* was found to depend on a promoter of its inserted gene.

In this study we sought to prescreen the cell lines having genes that are expressed in the developing nervous system.

MATERIALS AND METHODS

Construction of the gene trap vector

DNA fragments used for construction of pS*neo*N*lacZ*A (Fig. 1a) and pMoS*neo*N*lacZ*A (Fig. 1b) were as follows: (1) a 660-bp fragment containing a splice acceptor site of the mouse *E-cadherin* gene, (2) and 800-bp fragment encoding *Escherichia coli neo* gene from pMC1*neo*-polyA(-) (Stratagene, USA), (3) *lacZ* gene cassette

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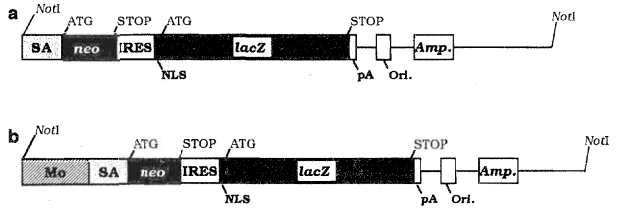

Fig. 1. Schematic diagram of the vectors used for this study, pS*neo*IN*lacZ*A (a) and pMoS*neo*IN*lacZ*A (b). The vector was digested with *Not*I and linearized. SA, mouse E-cadherin splice acceptor site; *neo*, *E. coli* neomycin phosphotoransferase gene; IRES, internal ribosomal entry site from encephalomyocarditis virus; NLS, nuclear localization signal from SV40 large T; *lacZ*, *E. coli* β-galactosidase gene; pA, polyadenylation signal of *tk* gene; Ori., replication origin in *E. coli*; *Amp.*, ampicillin resistant gene; ATG, initiation codon; STOP, stop codon; Mo, Moloney murine leukemia virus long-terminal repeat.

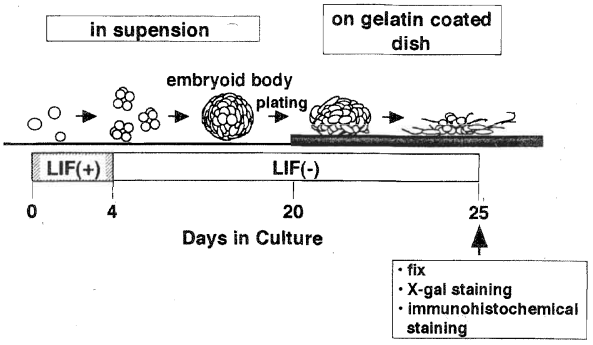

Fig. 2. Schematic presentation of the experimental protocol used for *in vitro* differentiation of ES cells.

whose eight amino acid residues of N-terminal were replaced by the nuclear localization signal (NLS) from SV40 large T, (4) a 600-bp fragment IRES derived from encephalomyocarditis virus (a gift from H.-S. Shin), (5) Moloney murine leukemia virus long-terminal repeat and (6) oligonucleotide containing a *Not*I site was inserted into the polylinker of pSPT19 (Pharmacia Biotech, USA). The above DNA fragments were ligated to the *Not*I-*Xba*I site as shown in Fig. 1.

To examine whether this gene trap vector can function in cells, the transfection experiments were performed by using C6 cells derived from rat glioma.

Cell culture, electroporation and selection

J1 ES cells (a gift from R. Jaenisch) were cultured on X ray-irradiated (2500 rad) mouse embryonic fibroblast (MEF) cells as a feeder in ES medium. The culture medium was *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-buffered Dulbecco's modified Eagle's medium (HEPES-buffered DMEM, GIBCO BRL, USA) supplemented with 15% heat-inactivated fetal bovine serum (ICN, USA), 0.1 mM non-essential amino acids (GIBCO BRL, USA), 0.1 mM 2-mercaptoethanol (SIGMA, USA), 1 mM sodium pyruvate (GIBCO BRL, USA), 500 U/ml leukaemia inhibitory factor (LIF; ESGRO, GIBCO BRL, USA) and penicillin-streptomycin as described previously (Satokata and Maas, 1994).

Rapidly growing ES cells were trypsinized, counted, washed and resuspended in electroporation buffer containing 20 mM HEPES (pH 7.0), 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose, and 0.1 mM 2-mercaptoethanol at 2.0×10^7 cells / 0.8 ml. The cell suspension was placed in an electroporation cuvette and gently mixed with the trap vector linearized with *Not*I. Cells were exposed to a single pulse at 400 V, 25 μF with a Bio-Rad Gene Pulser. They were then left in the electroporation buffer for 10 min at room temperature and then seeded onto X ray-irradiated MEF cells at 2.0×10^6 cells / 100 mm plate. Selection with G418 at 200 μg/ml (active dose; SIGMA, USA) was started 24 hr after electorporation. After 7 days of selection, independent G418-resistant colonies were picked up by using micropipettes. Each clone was trypsinized and expanded for freezing in liquid nitrogen and for further use. An aliquot of cells was replated in a 24-well tissue culture plate for observation of β-galactosidase activity.

In vitro differentiation

At first, ES cells were treated with 0.25% trypsin and cultured in suspension without feeder cells by using ES medium (Fig. 2). On day 4, ES medium was changed to LIF-free DMEM containing 25 mM HEPES (pH 7.3), 10% heat-inactivated fetal bovine serum and penicillin-streptomycin. The culture medium was changed every 4 days. Within 4-5 days, the cell clusters expanded three-dimensionally to form simple cystic embryoid bodies. After 20 days these cell clusters were transferred to gelatin-coated dishes to promote differentiation. On day 25, cells were examined by X-gal staining and immunohistochemistry.

X-gal staining and immunohistochemistry

Cells were fixed in 0.2% glutaraldehyde in PBS for 5 min at room temperature. After washing three times in PBS at room temperature, fixed cells were overlaid for 4 hr or overnight at 37°C in PBS containing 0.1% 4-chloro-5-bromo-3-indolyl-D-galactopyranoside (X-gal), 5 mM potassium ferricyanide ($K_3Fe(CN)_6$), 5 mM potassium ferrocyanide ($K_4Fe(CN)_6$), 1 mM $MgCl_2$, 0.01% deoxycholate, and 0.02% NP-40 in PBS. The cells were then rinsed several times in PBS and postfixed in 4% paraformaldehyde in PBS for immunohistochemical staining.

To identify neurons, neuron-glia precursor cells and glial cells, the differentiated ES cells were further stained with the following antibodies. Mouse monoclonal anti-microtubule associated protein (MAP2) (1:100; Boehringer Manheim GmbH, no. 1284959) and rabbit polyclonal anti-150-kDa neurofilament protein (NFP 150 kD) (1:5000) (Maeda *et al.*, 1987) were used as primary antibodies for immunocytochemistry for neurons. For neuron-glia precursor cells, rabbit polyclonal anti-nestin (1:1000; a gift from Y. Tomooka) was used. Glial cells were identified by the use of mouse monoclonal anti-glial fibrillary acidic protein (GFAP) (1:1000; Boehringer Manheim GmbH, no. 814369).

Generation and analysis of chimeric embryos

Blastocyst injection was performed according to the method described by Stewart (1993) and Kim *et al.*, (1992). Blastocysts (3.5 days postcoitum, dpc) were isolated from superovulated C57BL/6J female mice mated with males of the same strain. The embryos were kept in drops of HEPES-buffered DMEM supplemented with 10% fetal calf serum covered with light mineral oil (Fisher Chem, USA) in humidified CO_2 incubator (37°C, 5% CO_2). Usually, 10 ES cells were injected into each blastocoel. After injection, embryos were tranferred into the uterine horn of pseudopregnant ICR recipient female mice that were mated 2.5 days before with vasectomized males.

Chimeric embryos were recovered at 14.5 dpc. The day of plug formation of the pseudopregnant recipient mother was designated as 0.5 dpc. Embryos were fixed overnight at 4°C in a mixture of 0.2% glutaraldehyde, 1% paraformaldehyde and 0.02% NP-40 in PBS, followed by the demonstration of X-gal activity as described above except for staining temperature at 30°C.

RESULT

lacZ expression in G418-resistant C6 cells

In order to examine whether the gene trap vector, pS*neo*IN*lacZ*A, consisting of a promoterless *neo* gene followed by the IRES-NLS-*lacZ* can function in cells, the vector was integrated into C6 cells by electroporation. In case of integration of the vector into the transcription active unit of the C6 cell genome, they grew in the presence of G418 and formed the colony after culture for 14 days. Upon X-gal staining, about 62% of the G418-resistant colonies showed β-galactosidase activity (data not shown). About 79% of the G418-resistant colonies were stained with X-gal (data not shown) when C6 cells were transfected with a vector, pMoS*neo*IN*lacZ*A (Fig. 1b). This vector contains a Moloney murine leukemia virus long-terminal repeat (Mo). These results indicate that a dicistronic mRNA is transcribed from the integrated vector in C6 cell clones and that both of the cistrons are translated to produce functional proteins. In addition, X-gal staining was shown in nucleus under the control of the NLS from SV40 large T (Garcia *et al.*, 1991) which took the place of the eight amino acid residues of N-terminal of lacZ.

Screening of the gene trapped ES cells

About 600 independent G418-resistant ES cell colonies were obtained and were tested for *lacZ* expression. Of these, 10 colonies, designated as GT3-2, 3, 4, 5, 6, 7, 8, 11, 12, and 13, cultured on MEF cells with LIF were stained positively with X-gal (Fig. 3). The intensity of staining varied among the different ES cell lines obtained. For example, the majority of GT3-11 cells forming colonies exhibited intense X-gal staining (Fig. 3b). On the other hand, in GT3-8 and 12 weakly stained cells were predominant (Fig. 3a, c).

In vitro differentiation

It is hard to analyze the gene regulation under different ways of transcriptional control if the promoterless vector, pS*neo*IN*lacZ*A, was integrated in the multiple sites. For *in vitro* differentiation, we selected the cell lines in which the vector was integrated in a single locus of the genome. To determine the copy numbers, genomic DNA from each colony was analyzed by Southern blot hybridization (data not shown). Nine out of 10 X-gal positive cell lines except GT3-13 showed a single band. These 9 ES lines were subjected to *in vitro* differentiation experiment as described in the Materials and Methods.

From 2 to 5 days after transferring the cystic embryoid bodies to gelatin-coated dishes without LIF they developed into different types of tissues (Fig. 2). Cardiac muscle-like fibers were most easily identified because of their rhythmical contraction. Neuronal cells manifested the characteristic morphology and connection of the extended neurites to other cells. Based on immunohistochemical examination of these cells, three interesting cell lines (GT3-8, 11 and 12) were obtained.

In the differentiated GT3-8 cell line, many X-gal stained cells exhibited β-galactosidase activity. The *lacZ* expression remarkably increased in intensity as compared with that of undifferentiated GT3-8 cells. Some of them were found to produce neuronspecific proteins (NFP 150 kD and MAP2) and nestin (Fig. 4a, b, c). Although many GFAP-positive cells were seen, none of them had β-galactosidase activity. Lots of cells stained with X-gal displayed different morphologies, indicating that the trapped gene expresses not only in the nervous tissue but also in other tissues.

In the differentiated GT3-11 cell line, the number of X-gal stained cells was less than that in GT3-8 and GT3-12, and the characteristic neurites were failed to be found. Some of the GFAP- and nestin-positive cells showed β-galactosidase activity (Fig. 4d, e), but no nuclei of NFP- and MAP2-positive cells were stained with X-gal.

In the differentiated GT3-12 cell line, β-galactosidase activity was seen in many cells. A part of MAP2 producing cells showed weak β-galactosidase activity (Fig. 4f). Though many GFAP-positive cells were observed, they were invariably X-gal negative.

Expression of lacZ in the chimeric embryos

We analyzed the gene expression involved in the early

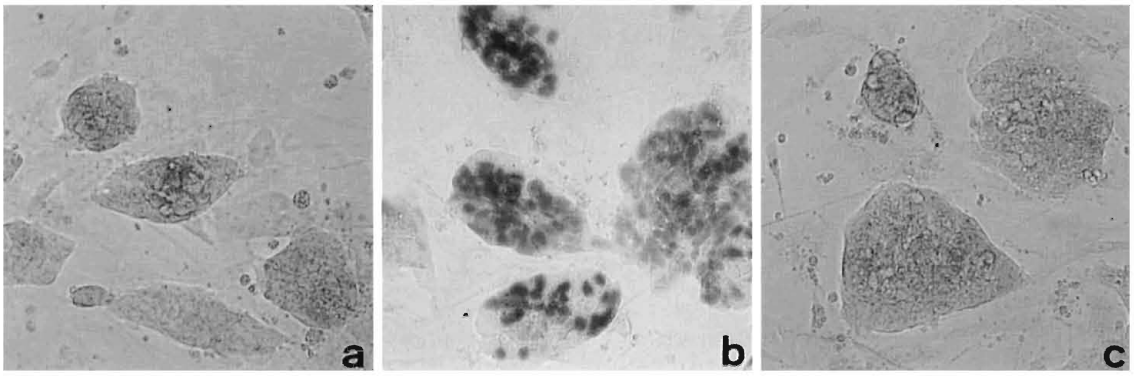

Fig. 3. Photomicrographs showing expression of *lacZ* in undifferentiated ES cell colonies from cell lines GT3-8 (a), GT3-11 (b) and GT3-12 (c). Cells were cultured in ES medium with LIF for 2 days. β-galactosidase activity (blue). ×97.

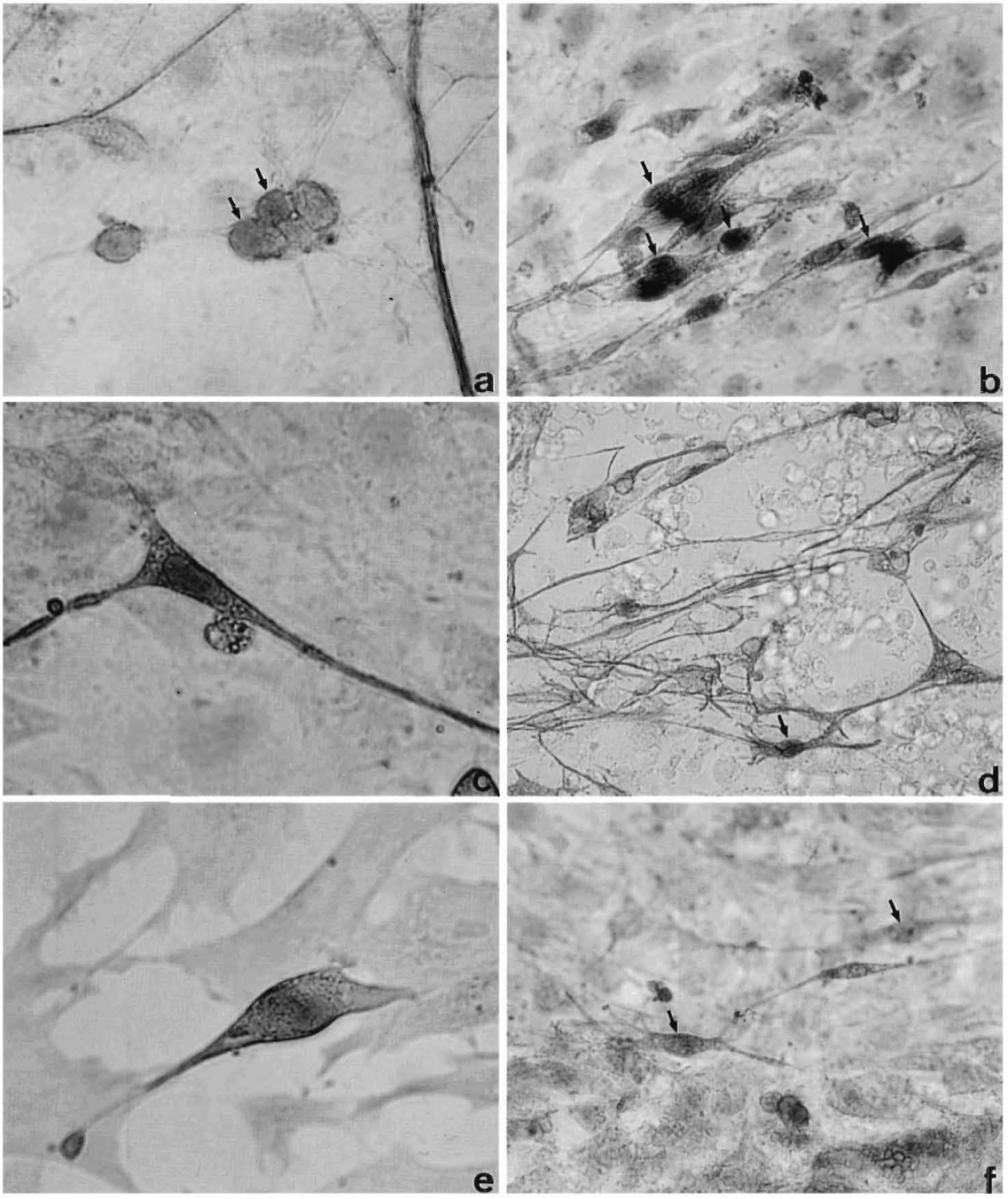

Fig. 4. Photomicrographs showing neuronal cells in ES cell lines GT3-8 (a, b and c), GT3-11 (d and e) and GT3-12 (f) replated on gelatin-coated dishes after suspension culture in the absence of LIF. After staining with X-gal (blue), the same culture plates were immunohistochemically reacted with antibodies against neuronspecific (a, NFP 150 kD; b and f, MAP2), glia specific proteins (d, GFAP) or nestin (c and e) (brown). a, c and e: ×380. b, d and f: ×190. Arrows indicate immunoreactive cells (brown) whose nuclei are stained simultaneously with X-gal (blue).

development of the central nervous system (CNS) by using promoterless *lacZ* as a reporter gene. The chimeric embryos from the GT3-8, GT3-11 and GT3-12 cell lines were collected at 14.5 dpc and stained with X-gal. In all three strains, β-galactosidase activity was observed in the nervous system (Fig. 5). This activity was concentrated in nuclei. These *lacZ* expression patterns are consistent with what we observed in the differentiated ES cells *in vitro*.

In the GT3-8 strain, the β-galactosidase activity was observed ubiquitously and more intensely than the activity observed in the ES cells (Fig. 5a, b). Strong staining was seen in the heart, tongue, lower lip and vertebra. On the other hand, relatively weak β-galactosidase activity was seen throughout the fetal brain (Fig. 5c).

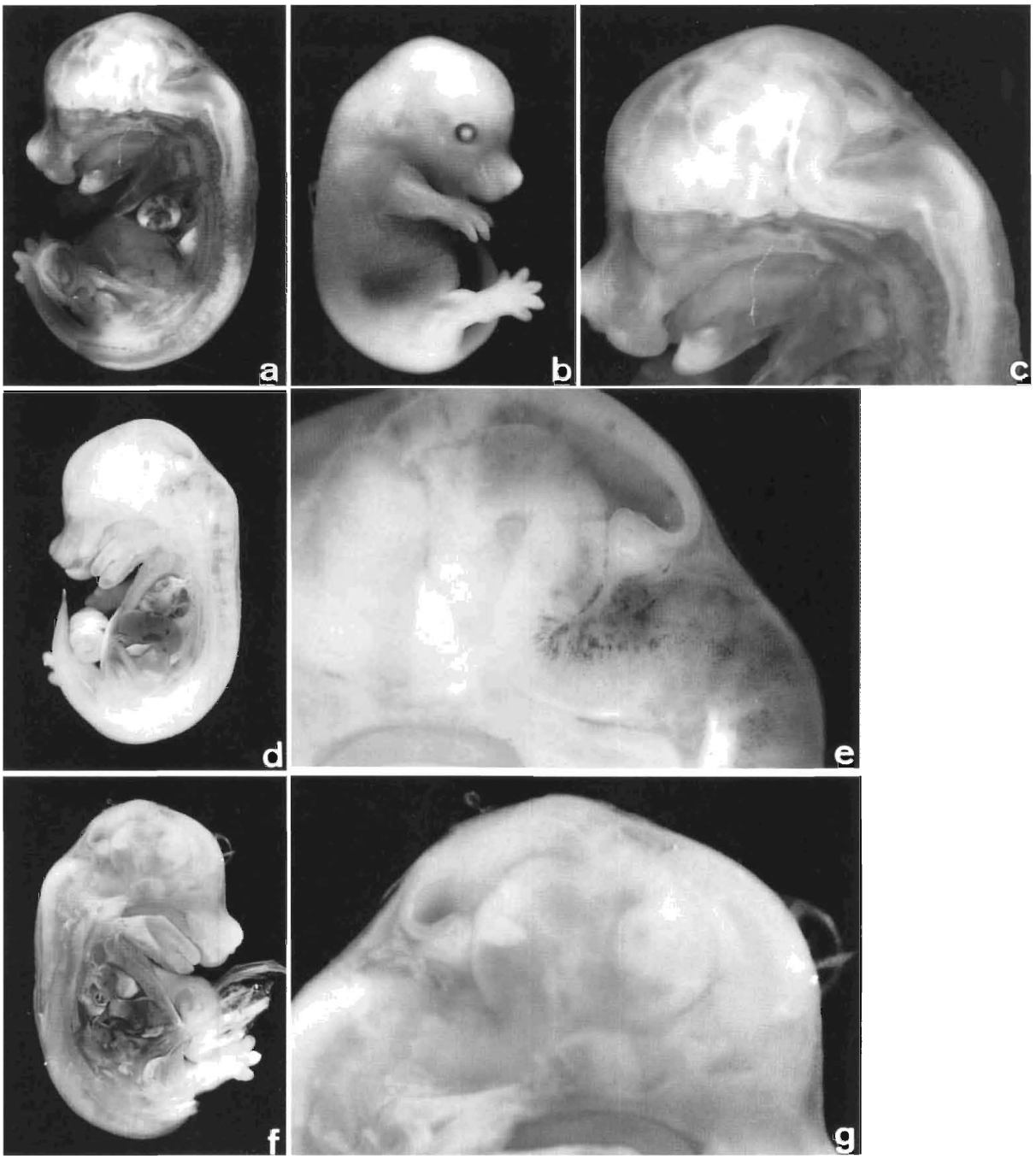

Fig. 5. Photographs showing X-gal staining patterns of 14.5 dpc mouse chimeric embryos derived from GT3-8 (a, b and c), GT3-11 (d and e) and GT3-12 (f and g). *lacZ* activity is detected in sagittal sections of bodies (a, d, and f), sagittal sections of heads (c, e and g) and the whole embryo (b). a, b, d and f: ×4. c: ×8. e: ×16. g: ×12.

The GT3-11 derived chimeric embryos displayed a well defined pattern of X-gal staining (Fig. 5d, e). Strongly reacted cells were detected at the ventral part of the midbrain, pons and spinal cord. We also observed some X-gal stained cells in the roof of the caudal part of midbrain, epithelial choroid layer, and dorsal root ganglions. In other tissues, strong reaction was seen throughout the heart. These staining patterns were observed in all chimera produced from GT3-11 in this study. Such clear distribution of the *lacZ* gene expression indicates a tissue specific regulation of this reporter gene by the trapped gene.

In GT3-12-derived chimeric embryos, a moderate level of *lacZ* expression was found in the central nervous system. The reaction product was not confined to neural tissue (Fig. 5f, g).

In order to confirm whether the three ES cell lines with

the trapped genes contribute to the germ line, the chimeras were bred further with C57BL/6J mice. The GT3-11 and GT3-12 cell lines, but not GT3-8 were transmitted into the germ line.

DISCUSSION

Neurones and glial cells form a complex net work that is characterized by a high degree of regional diversity in terms of both function and morphology. The mechanism as to how this diversity develops is a key issue in modern neurobiology. Recently, an increasing number of investigators have been challenging to solve this problem in transgenic model mice (Snider, 1994).

One approach, gene targeting, involves homologous replacement of the structurally characterized gene followed by various mutagenesis. By using this technique, important genes involved in morphogenesis of the brain are hitherto clarified (Carpenter *et al.*, 1993; Dollé *et al.*, 1993; Hanks *et al.*, 1995; Joyner *et al.*, 1991; Mark *et al.*, 1993; Schneider *et al.*, 1993; Wurst *et al.*, 1994). In these gene targeting experiments, DNA probes are prepared on the basis of known protein structures of the animal used, or according to the sequence similarity to those genes isolated from mutant organisms of other species.

Gene trapping as another approach has also been applied to mutagenic experiments (Friedrich and Soriano, 1991; Gossler *et al.*, 1989; Joyner *et al.*, 1992; Niwa *et al.*, 1993; Skarnes *et al.*, 1992; Takeuchi *et al.*, 1995). Unlike the gene targeting method, gene trapping permits random insertion of genes into the genome, which offers the advantage for identifying novel genes. By using such gene trapping technique, Takeuchi *et al.* (1995) identified a new gene *"jmj"* that is expressed in a spatially defined domain at the midbrain-hindbrain boundary and in the cerebellum. The fetal mice homozygous for the mutation in *jmj* died in utero and showed a defect in the closure of the neural tube. Chen *et al.* (1994) also established a new mutant mouse strain, ROSAgeo5, carrying a mutation in the transcriptional enhancer factor 1 (TEF-1) gene by employing a retroviral gene trap. Their report is the first to define that TEF-1 plays an essential role in the maturational stage of cardiogenesis.

To date efforts for improving the gene trapping method have been centered on identifying as many new genes as possible (Friedrich and Soriano, 1991; Gossler *et al.*, 1989; Niwa *et al.*, 1993; Skarnes *et al.*, 1992; Takeuchi *et al.*, 1995). In this study we made an effort to develop a more efficient way to obtain ES cells whose handled genes are expressed in a restricted tissue. Reddy *et al.* (1992) compared β-galactosidase activity of undifferentiated and differentiated ES cells in the course of culture and its activity after introducing them into chimeras. In our study, too, *in vitro lacZ* expression was found to persist in chimeric animals as well. Taking advantage of this expression of the reporter gene, we prescreened gene trapped ES cell line. Such prescreening step is thought to reduce the time required for anlaysis of genes in mutant mice.

In this study, we observed neuron-glia precursor cells (nestin-positive cells), post-mitotic neurons (NFP 150 kD-positive cells) and post-mitotic glial cells (GFAP-positive cells) at the same time. This is not surprising in view of the *in vivo* evidence that neuron-glia precursor cells are observable among already differentiated cells in the CNS of perinatal mice (Dahlstrand *et al.*, 1995).

Differentiated GT3-8 cells were found to exhibit a higher level of β-galactosidase activity in contrast to its low activity in the undifferentiated state. In GT3-8 derived chimeric embryo, the β-galactosidase activity was more intense than in ES cells *in vitro*. Perhaps the expression level of a trapped gene in GT3-8 became higher as development proceeded. We regard our *in vitro* differentiation experiment as a useful method to identify a gene whose expression level becomes higher toward the late developmental stage.

It seems of interest to note that in GT3-11 cells X-gal staining was restricted to the brain and heart. Wen *et al.* (1992) has identified a novel gene referred to as neuregulin that is involved in the development of both the brain and heart. Fetal mice lacking neuregulin or their receptors (erbB4 and erbB2) die during the mid-gestational period owing to the aborted development of myocardial trabeculae in the heart ventricle (Gassmann *et al.*, 1995; Lee *et al.*, 1995; Meyer and Birchmeier, 1995). They also displayed striking alterations in innervation of the hindbrain in the CNS. In view of these results, it is tempting to postulate that this gene has some to do with our trapped gene in GT3-11 cell lines.

ES cells spontaneously start to differentiate to various cell types, if they are allowed to reach a high density in monolayers or to form aggregates in suspension without LIF. For example, ES cells were differentiated to parietal and visceral endoderm, cardiac muscle, and other unidentified types of cell (Doetschman *et al.*, 1985). Neuronal differentiation of ES cells was shown to occur spontaneously when ES cells were maintained in suspension culture and then implanted into adult nude mice, either subcutaneously or under the kidney capsule (Chen and Kosco, 1993). They reported that the highest frequency of gross differentiated ES structures developed when ES cells were cultured in suspension for 2-3 weeks. Our aim to select ES cell clones possessing the neural tissue-like nature was attained by performing suspension culture for 20 days. However, our culture system was not devised as to permit ES cells to differentiate specifically into neural cells. In view of the recent observation that neurons and glial cells appeared more frequently in cultures after adding retinoic acid (Fraichard *et al.*, 1995), further devices of the culture method are currently in progress.

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REFERENCES

Capecchi MR (1989) Altering the genome by homologous recombination. Science 244: 1288–1292

Carpenter EM, Goddard JM, Chisaka O, Manley NR, Capecchi MR (1993) Loss of Hox-A1 (Hox-1.6) function results in the reorganization of the murine hindbrain. Development 118: 1063–1075

Chen U, Kosco M (1993) Differentiation of mouse embryonic stem cells in vitro: III. Morphological evaluation of tissues developed after implantation of differentiated mouse embryoid bodies. Dev Dyn 197: 217–226

Chen Z, Friedrich GA, Soriano P (1994) Transcriptional enhancer factor 1 distruption by a retroviral gene trap leads to heart defects and embryonic lethality in mice. Genes Dev 8: 2293–2301

Dahlstrand J, Lardelli M, Lendahl U (1995) Nestin mRNA expression correlates with the central nervous system progenitor cell state in many, but not all, regions of developing central nervous system. Dev Brain Res 84: 109–129

Doetschman TC, Eistetter H, Katz M, Schmidt W, Kemler R (1985) The *in vitro* development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. J Embryol Exp Morph 87: 27–45

Dollé P, Lufkin T, Krumlauf R, Mark M, Duboule D, Chambon P (1993) Local alterations of Krox-20 and Hox gene expression in the hindbrain suggest lack of rhombomeres 4 and 5 in homozygote null Hoxa-1 (Hox-1.6) mutant embryos. Proc Natl Acad Sci USA 90: 7666–7670

Fraichard A, Chassande O, Bilbaut G, Dehay C, Savatier P, Samarut J (1995) *In vitro* differentiation of embryonic stem cells into glial cells and functional neurons. J Cell Sci 108: 3181–3188

Friedrich G, Soriano P (1991) Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. Genes Dev 5: 1513–1523

Garcia BJ, Heitman J, Hall MN (1991) Nuclear protein localization. Biochim Biophys Acta 1071: 83–101

Gassmann M, Casagranda F, Orioli D, Simon H, Lai C, Klein R, Lemke G (1995) Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. Nature 378: 390–394

Gossler A, Joyner AL, Rossant J, Skarnes WC (1989) Mouse embryonic stem cells and reporter constructs to detect developmentally regulated genes. Science 244: 463–465

Hanks M, Wurst W, Anson CL, Auerbach AB, Joyner AL (1995) Rescue of the *En-1* mutant phenotype by replacement of *En-1* with *En-2*. Science 269: 679–682

Jang SK, Wimmer E (1990) Cap-independent translation of encephalomyocarditis virus RNA: structural elements of the internal ribosomal entry site and involvement of a cellular 57-kD RNA-binding protein. Genes Dev 4: 1560–1572

Joyner AL, Herrup K, Auerbach BA, Davis CA, Rossant J (1991) Subtle cerebellar phenotype in mice homozygous for a targeted deletion of the En-2 homeobox. Science 251: 1239–1243

Joyner AL, Auerbach A, Skarnes WC (1992) The gene trap approach in embryonic stem cells: the potential for genetic screens in mice. Ciba Found Symp 165: 277–288

Kim DG, Kang HM, Jang SK, Shin HS (1992) Construction of a bifunctional mRNA in the mouse by using the internal ribosomal entry site of the encephalomyocarditis virus. Mol Cell Biol 12: 3636–3643

Lee KF, Simon H, Chen H, Hauser C (1995) Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature 378: 394–398

Maeda T, Iwanaga T, Fujita T, Takahashi Y, Kobayashi S (1987) Distribution of nerve fibers immunoreactive to neurofilament protein in rat molars and periodontium. Cell Tissue Res 249: 13–23

Mansour SL, Thomas KR, Capecchi MR (1988) Disruption of the protooncogene *int-2* in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. Nature 336: 348–352

Mark M, Lufkin T, Vonesch JL, Ruberte E, Olivo JC, Dollé P, Gorry P, Lumsden A, Chambon P (1993) Two rhombomeres are altered in *Hoxa-1* mutant mice. Development 119: 319–338

Meyer D, Birchmeier C (1995) Multiple essential functions of neuregulin in development. Nature 378: 386–390

Niwa H, Araki K, Kimura S, Taniguchi S, Wakasugi S, Yamamura K (1993) An efficient gene-trap method using poly A trap vectors and characterization of gene-trap events. J Biochem 113: 343–349

Reddy S, Rayburn H, von Merchner H, Ruley HE (1992) Fluorescence-activated sorting of totipotent embryonic stem cells expressing developmentally regulated *lacZ* fusion genes. Proc Natl Acad Sci USA 89: 6721–6725

Satokata I, Maas R (1994) Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. Nature Genet 6: 348–356

Schneider MS, Topilko P, Seitandou T, Levi G, Cohen TM, Pournin S, Babinet C, Charnay P (1993) Disruption of *Krox-20* results in alteration of rhombomeres 3 and 5 in the developing hindbrain. Cell 75: 1199–1214

Skarnes WC, Auerbach BA, Joyner AL (1992) A gene trap approach in mouse embryonic stem cells: the lacZ reported is activated by splicing, reflects endogenous gene expression, and is mutagenic in mice. Genes Dev 6: 903–918

Snider WD (1994) Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. Cell 77: 627–638

Stewart CL (1993) Production of chimeras between embryonic stem cells and embryos. In "Guide to Techniques in Mouse Development" Ed by PM Wassarman, ML DePamphilis, Academic Press, San Diego, pp 823–855

Takeuchi T, Yamazaki Y, Katoh FY, Tsuchiya R, Kondo S, Motoyama J, Higashinakagawa T (1995) Gene trap capture of a novel mouse gene, *jumonji,* required for neural tube formation. Genes Dev 9: 1211–1222

Wen D, Peles E, Cupples R, Suggs SV, Bacus SS, Luo Y, Trail G, Hu S, Silbiger SM, Levy RB, Koski RA, Lu HS, Yarden Y (1992) Neu differentiation factor: a transmembrane glycoprotein containing and EGF domain and an immunoglobulin homology unit. Cell 69: 559–572

Wurst W, Auerbach AB, Joyner AL (1994) Multiple developmental defects in *Engrailed-1* mutant mice: an early mid-hindbrain deletion and patterning defects in forelimbs and sternum. Development 120: 2065–2075

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